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# Biological control of citrus canker pathogen *Xanthomonas citri* subsp. *citri* using Rangpur lime endophytic bacteria

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## Abstract

**Background:** Rangpur lime (RL) is a common rootstock cultivar used worldwide. However, it is known to have a high susceptibility to citrus canker (CC). To meet the increasing demand for healthy citrus seedlings, this research aimed to study the potency of RL endophytic bacteria as a biocontrol agent of the CC pathogen *Xanthomonas citri* subsp. *citri*. The isolates were collected from healthy RL leaves and subjected to in vitro and in planta antagonistic tests against XCC, alongside its cell-free supernatant (CFS). Potential isolates were identified according to their 16S rDNA sequence similarities.

**Results:** As many as 21 isolates were obtained from the leaves of healthy RL trees. Two (B1 and C8) isolates demonstrated promising inhibitory activity against XCC. Based on the in vitro assays, the cell suspensions (CS) of these isolates could effectively inhibit the growth of XCC, with an optimum clear zone diameter of 8.41 and 7.51 mm, respectively. Consistent with CS, their CFSs also displayed similar antagonistic potencies against XCC with the highest clear zone recorded being 7.23 mm and 6.22 mm, respectively. Further investigations revealed that the minimum inhibitory concentration of B1 and C8 CFSs was 25 µg/ml. It was also found that both CFSs were sensitive to high heat and very low pH, but stable in the presence of proteinase-K. In the in vivo assay, both CS and CFS treatments of B1 and C8 could effectively protect the Rangpur lime from CC. Treated plants had significantly lower disease incidences and developed less severe symptoms than the control plants. Based on their 16S rDNA sequence, B1 and C8 were identified as *Staphylococcus pasteuri* and *Staphylococcus warneri*, respectively.

**Conclusion:** It was concluded that *Staphylococcus pasteuri* and *Staphylococcus warneri* could be promising biocontrol agents of XCC. These bacterial isolates can be used as an alternative prevention measure to CC as opposed to commonly used chemicals.

**Keywords:** Biological control, Citrus canker, Endophytic bacteria, *Staphylococcus pasteuri*, *Staphylococcus warneri*, *Xanthomonas citri* subsp. *citri*

## Background

Citrus canker (CC) is one of the most destructive, contagious, and pervasive citrus diseases in the world caused by phytopathogenic bacteria *Xanthomonas citri* subsp.

*citri* (XCC). It is commonly found in various citrus-growing regions all over the world, where it has caused a major decline in citrus production and marketability (Behlau et al. 2010). These declines are primarily attributed to the symptoms caused by the disease. Brown and corky necrotic lesions, which usually appear as a result of perpetual hyperplasia induced by the pathogens, can significantly impair the visual of the fruits and reduce their economic value in the fresh market. On the other hand, other physiological symptoms such as premature

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fruit drop, twigs dieback, defoliation, and stunting can severely affect the overall fruit production and plant longevity (Gottwald et al. 2002).

Rangpur lime (*Citrus limonia*, Osbeck) is one of the cultivars that are prone to citrus canker infection with a 56.3% incidence rate (Stover et al. 2014). Despite being highly susceptible to citrus canker, it is widely cultivated in citrus-growing countries as a rootstock in grafting due to its high tolerance to drought stress, and resistance to tristeza disease. Moreover, its popularity is also attributed to other favorable traits such as early maturation of fruits, vigorous trees, and heavy bearings, making it the perfect rootstock in dry and semidry soils (Morelli et al. 2019).

Since rootstock quality and availability hold a vital part in maintaining a sustainable citriculture, safety and prevention measures need to be taken to protect Rangpur lime from the threat of CC. Some of the common protocols are antibiotic spray and copper spray, which are usually applied before the rainy seasons as a prevention measure against citrus canker outbreaks. These methods are commonly used in Indonesia, Brazil, and the USA (Gottwald et al. 2002). In addition, in some areas in Brazil and the USA, several alternatives such as systemic acquired resistance (SAR) inducers and windbreaks are also employed to boost tree resistance and halt the dispersal of CC (Graham and Myers 2013).

Despite yielding successful results in eliminating CC, these methods have several drawbacks in terms of their sustainability and physiological cost. Constant use of chemical compounds like copper may lead to the selection of newly resistant strains (Behlau et al. 2013), and their accumulations on soil may exert a phytotoxic effect on the citrus trees (Alva et al. 1995). Furthermore, their protective abilities are not long-lasting and can be easily undermined by wind-driven rain that usually sweeps the chemicals from the targeted areas and exposes the pathogen to the mean of entrances (Gottwald and Timmer 1995). On the other hand, other prevention methods such as systemic acquired resistance (SAR) inducers are not relatively economical and can easily impose deleterious effects on plants if not applied properly (Walters and Heil 2007).

Endophytic bacteria can serve as an alternative method to control plant pathogens such as XCC. It controls pathogens by producing secondary metabolites and competing for nutrients and space (Morales-Cedeño et al. 2020). Previous findings have shown the biological control activities of endophytic bacteria against several *Xanthomonas* species. For instance, *Pseudomonas fluorescens* SF4c and its secondary metabolite, talocin, are found to control *Xanthomonas vesicatoria* in tomatoes (Principe et al. 2018). *Pseudomonas aeruginosa* BRp3 also

demonstrated biocontrol activity to *X. oryzae* pv. *oryzae* in Super Basmati rice variety in Pakistan. Its controls *X. oryzae* pv. *oryzae* by producing cell-free supernatant (CFS) that contains multiple antibacterial compounds such as siderophores, rhamnolipids, 4-hydroxy-2-alkylquinolines (HAQs), 2,3,4-trihydroxy-2-alkylquinolines, and 1,2,3,4-tetrahydroxy-2-alkylquinolines (Yasmin et al. 2017).

The use of living cells or cell suspensions (CS) has received enormous attention worldwide. However, albeit its success, the effectiveness and the formulations of CS for field applications still possess major challenges due to their instability. Without proper or sufficient carrier, the cell within the suspension could die easily when introduced to the ecosystem. This lack of durability, combined with other factors such as competition with other microflora and several environmental conditions, makes it difficult to accumulate the biocontrol agent in the targeted area (Bashan et al. 2014). As a result, only a few types of CS biocontrol agents circulate on the market. Most of them are still only described in the scientific literature or used in a very controlled environment (Pellegrini et al. 2020).

These challenges provide the need to formulate a CFS biocontrol agent. As opposed to CS, CFS is known to be relatively more stable in the field and may exert antagonistic potencies similar to CS (Morel et al. 2016). One of the most researched CFS as a biocontrol agent is the CFS from *Bacillus amyloliquefaciens*. Its CFS consisted of some promising antimicrobial substances such as iturin, surfactin, amylocyclin, and fengycin that have been known to control various types of plant pathogens such as *X. campestris* pv. *campestris* (Da Silva et al. 2018).

In the case of CC, studies on the use of beneficial bacteria and its CFS are still relatively limited and heavily focused on the use of rhizospheric bacteria rather than its endophytic counterparts. Therefore, to help meet the continuous demand for citrus canker-free Rangpur lime plants and provide alternative biocontrol agents of citrus canker, this study was conducted to evaluate the potency of Rangpur lime endophytic bacteria and its secondary metabolite (in the form of CFS) as the biological control agent of *Xanthomonas citri* subsp. *citri* subsp. *citri* (XCC).

## Methods

### Sample surface sterilization and endophytic bacteria isolation

Healthy leaves of Rangpur lime were obtained from the Punten Citrus Experimental Garden Facility in Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI) (Batu, East Java, Indonesia) and surface-sterilized according to Araujo et al. (2002). Briefly, as many as 25 g of healthy leaves were cleaned in tap water to

remove adherent particles. The leaf samples were then dipped into 70% ethanol solution for 5 min, 2% sodium hypochlorite solution (NaClO) for 5 min, and 70% ethanol solution for 30 s. The samples were rinsed twice in sterile water for 2 min.

The isolation of endophytic bacteria was conducted according to Costa et al. (2011). Surface-sterilized leaves were cut into small fragments and pounded with a sterilized mortar and pestle. As many as 25 g of smooth leaves then was suspended in 225 ml physiological salt solution and diluted in serial dilutions until  $10^{-6}$  dilutions were obtained. Then, 100  $\mu$ l of each serial dilution was inoculated onto nutrient agar (NA) with a Drigalski rod and incubated at 30 °C for 48 h. Bacterial colonies were counted and purified.

#### Preparation of XCC culture

XCC used in this study was obtained from the Plant Pathology Laboratory at the Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI). The bacterium was subcultured in nutrient agar (NA) and kept in 20% glycerol at  $-80$  °C for long-term storage. For the research, XCC was prepared by cultivating the pathogen in the nutrient broth (NB) at 30 °C until it had reached its exponential growth phase. In brief, one loopful of XCC inoculum was transferred into an NB medium and kept at 30 °C for 24 h under 120 rpm orbital shaking. Then, 10% of the previous XCC culture was transferred into a new NB medium and kept at 30 °C within a 120 rpm rotary shaker until it reached the exponential growth phase with  $10^6$  cell/ml density (approximately 12 h). This culture was then used for the antagonistic assay in both laboratory and field experiments.

#### Antagonistic assay of Rangpur lime endophytic bacteria against XCC

A total of 21 endophytic bacterial isolates were obtained from healthy Rangpur lime leaves and subjected to antagonistic assay against XCC for the initial screening according to Li et al. (2012) with minor alterations. Each isolate was cultured in NB at 30 °C for 24 h under 120 rpm orbital shaking until it reached 0.5 optical density (OD) ( $\lambda$ : 600 nm). Then, 5.0 ml of bacterial culture was transferred into a new NB and kept at 30 °C for 72 h in a 120 rpm rotary shaker. Into an XCC-inoculated (0.1 ml;  $10^6$  cell/ml density) NA plate, wells (6 mm diameter) were made using sterile cork borers and filled with 50  $\mu$ l of each bacterial culture. Sterile ddH<sub>2</sub>O was the negative control in this research. Before the incubation, plates were first kept at 4 °C for 2 h to allow culture absorption into the medium (Hamayun et al. 2021). Plates were then kept at 30 °C for 48 h, and the clear zone around each well was measured. Isolates that demonstrated antagonistic

activity against XCC were selected for further assay. This test was carried out in three replications based on a completely randomized design.

#### Effects of incubation time on the antagonistic activity of the selected bacteria against XCC

To further investigate the potency of two selected isolates, here we evaluate their antagonistic activities in response to different incubation periods. All selected isolates in this test were incubated for 168 h and subjected to an antagonistic assay against XCC. Firstly, each selected bacterial isolate was cultivated in an NB at 30 °C for 24 h with 120 rpm orbital shaking until it had reached 0.5 OD ( $\lambda$ : 600 nm). Then, as much as 5.0 ml of bacterial culture was inoculated into a new 45 ml of NB and kept at 30 °C for 168 h in a 120 rpm rotary shaker. Every 24 h, 50  $\mu$ l of bacterial culture was taken and inoculated into a well within an already XCC-inoculated (0.1 ml;  $10^6$  cell/ml density) NA plate. Sterile ddH<sub>2</sub>O served as a negative control in this test. Before the final incubation, plates were stored at 4 °C for 2 h to allow the culture diffusion to the medium. Finally, the plates were kept at 30 °C for 48 h, and a clear zone around each well was measured. Unlike the previous assay, this test was carried out in triplicates based on a complete block randomized design.

#### Antagonistic assay of endophytic bacteria secondary metabolites against XCC

Similar to the previous experiment, this test was performed to evaluate the effect of the incubation period on the antagonistic activity of the secondary metabolites. This experiment was conducted in triplicates according to Silva et al. (2018), with minor modifications. The secondary metabolites (in the form of cell-free supernatant (CFS)) were prepared by cultivating each bacterial isolate in an NB medium at 30 °C for 24 h in a 120 rpm orbital shaking. Then, 7.0 ml of the culture (optical density 0.5 ( $\lambda$ : 600 nm)) was transferred into a 63 ml new NB and incubated at 30 °C for 168 h on a 120 rpm orbital shaker. Afterward, 5 ml of each bacterial culture at the interval of 24 h was taken and centrifuged at 4000g for 10 min. Finally, the CFS of each endophytic bacterium was individually sterilized with a 0.22- $\mu$ m Millipore membrane and used in the antagonistic assay against XCC using the agar well diffusion method as above. Sterile ddH<sub>2</sub>O was used as the negative control in this research.

#### Secondary metabolite minimum inhibitory concentration (MIC) determination

The MIC value of the most effective secondary metabolite was evaluated according to the protocols proposed by Mostafa et al. (2018) with minor modifications. The secondary metabolite (in the form of cell-free supernatant)

was prepared according to the method described above and harvested at 168 h. It was then freeze-dried and subjected to several concentrations (400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml) through dilutions in sterile water. Each concentration of freeze-dried CFS solutions was prepared individually and sterilized using a 0.22-µm Millipore membrane. To determine the MIC, the agar well diffusion technique was used in triplicates, and sterile ddH<sub>2</sub>O was used as a negative control.

#### Physicochemical characterizations of secondary metabolite

The most promising CFS produced by each selected bacteria was characterized by their heat, pH, and proteinase-K stability. The secondary metabolites were harvested at 168 culture age and prepared according to the method mentioned above. The secondary metabolites were then individually treated with heat, pH, and proteinase-K. The effect of each treatment was evaluated with the agar well diffusion assay. Untreated CFSs were used as control. Each treatment was conducted in triplicates.

For pH stability, the CFS was adjusted to various pH ranges from 2 to 12 and kept at room temperature for 1 h. Before the inoculation in the agar well culture, the secondary metabolites were readjusted to their initial pH (for 7.53 B1 and 7.89 C8) (Biswas et al. 2017). For heat stability, the CFS was heated to a temperature ranging from 30 to 121 °C for 1 h. The secondary metabolites then were let cool to room temperature before the inoculation to agar well culture (Baharudin et al. 2021). To investigate the proteinase-K stability, 1 ml of the prepared CFS was digested with 15 µl of proteinase-K (1000 U/ml). It was then incubated at 30 °C for 1 h (Li et al. 2012).

#### In planta antagonistic assay of endophytic bacteria cell suspensions and secondary metabolites

Healthy Rangpur lime plants (5–6 months old, 30–40 cm in height) used in this experiment were obtained from the seedlings production facility in the Indonesian Citrus and Subtropical Fruits Research Institute. The plants were grown in a 7.5 × 30 cm polybag with ¾ total soil volume and placed in a screen house with 28 ± 2 °C temperature. During the experiments, all plants were watered with sterile distilled water every 2 days or when needed. The independent treatments in this experiment consist of cell suspension (CS) with a density of 10<sup>6</sup> cell/ml and CFS of the two selected endophytic bacteria (B1 and C8) harvested after 168 h of incubation. A negative control group was used in the experiment, where all the plants in this group were only treated with XCC. Six plants were used in each group, and ten healthy leaves were chosen for each plant. The leaves were chosen according to their size, color, and age similarities.

*In planta* inhibition tests were performed by inoculating the CS and CFS of the selected isolates onto the leaf surfaces. In brief, each selected leaf was individually wiped with a sterile tissue paper and then sprayed uniformly with 3 ml of 10<sup>6</sup> cell/ml endophytic bacterial CS or 3 ml CFS. The sprayed leaves are then covered with a plastic cover for 3 days. After 3 days, each selected leaf was then punctured with a sterile needle and sprayed uniformly with 10<sup>6</sup> cells/ml of XCC culture. After being treated with XCC, all plants were grown for 28 days. The disease progression over time was observed by counting the number of infected leaves with symptoms every 7 days and expressed as a percentage of overall selected leaves (Li et al. 2012). On the final day of the experiment, the number of necrotic spots and the percentage of the symptomatic leaf area were determined according to the equations proposed by Ellibox and Umaharan (2008). For the second and third parameters, six replications were used. Here, six plants (consisting of ten selected leaves/plants) from each treatment represent six replications.

#### Phylogenetic identification of the selected endophytic bacteria

Selected bacterial isolates were identified according to their 16S rDNA sequences. The chromosomal DNA of bacterial isolates was extracted according to William et al. (2012) and suspended in 40 µl TE buffer and stored at –20 °C. Firstly, each selected bacterial isolate (B1 and C8) was inoculated in NB medium and kept at 30 °C for 24 h. Then, 2 ml of each bacterial culture was taken and centrifuged at 12,000 rpm for 10 min. Afterward, the supernatant was removed and the pellet was resuspended with 567 µl of buffer tris–EDTA (TE) and 30 µl of 10% sodium dodecyl sulfate (SDS) and incubated at 37 °C for 1 h. After the incubation, 100 µl of 5 M sodium chloride (NaCl) and 80 µl of 1 M hexadecyltrimethylammonium bromide (CTAB) were added to the solution. Then, the solution was incubated at 30 °C for 10 min. The solution was then mixed with 750 µl of 24:1 chloroform/isoamyl alcohol (C/I) and centrifuged at 12,000 rpm for 5 min. Then, as much as 700 µl of supernatant was taken, mixed with 25:24:1 phenol/chloroform/isoamyl alcohol (PCI) solution, and centrifuged at 12,000 rpm for 5 min. Afterward, the supernatant was taken, mixed with 300 µl cold isopropanol, and centrifuged at 12,000 rpm for 5 min to allow DNA precipitation. Then, the DNA pellet was washed with 500 µl of 70% ethanol through centrifugation at 12,000 rpm for 5 min. The supernatant was removed, and the DNA pellet is resuspended in 40 µl TE and kept at –20 °C for long-term storage.

The amplification process was performed under the PCR technique with the universal primer of 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r

(5'-TACGGAGTTACCTTGTTAACGACTT-3'). The PCR mix cocktail contains a 5 µl DNA template, 2.5 µl of each 27f and 1492r primer, 25 µl PCR Master Mix, and 16 µl ddH<sub>2</sub>O. The amplification was performed under the program consisted of 1 cycle pre-denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and 1 cycle of post-extension at 72 °C for 1 min. The 16S rDNA amplicon was sequenced at Bioneer, South Korea, and aligned with reference bacterial sequences using Basic Local Alignment Tools (BLAST) in the National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed using MEGA7 software with the maximum likelihood method with the Tamura–Nei algorithm in 1000 bootstrap replications (Meena et al. 2015).

**Data analysis**

All statistical data analyses in this research were carried out in SPSS Ver. 25. Data from the initial screening, MIC and physicochemical characterizations, leaf symptoms area, and necrotic spots were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey HSD test. Meanwhile, data collected from the effect of incubation time on the selected isolates' antagonistic activities and secondary metabolites' antagonistic activities were analyzed using two-way ANOVA followed by the Tukey HSD test.

**Results**

**Antagonistic activity of Rangpur lime endophytic bacteria**

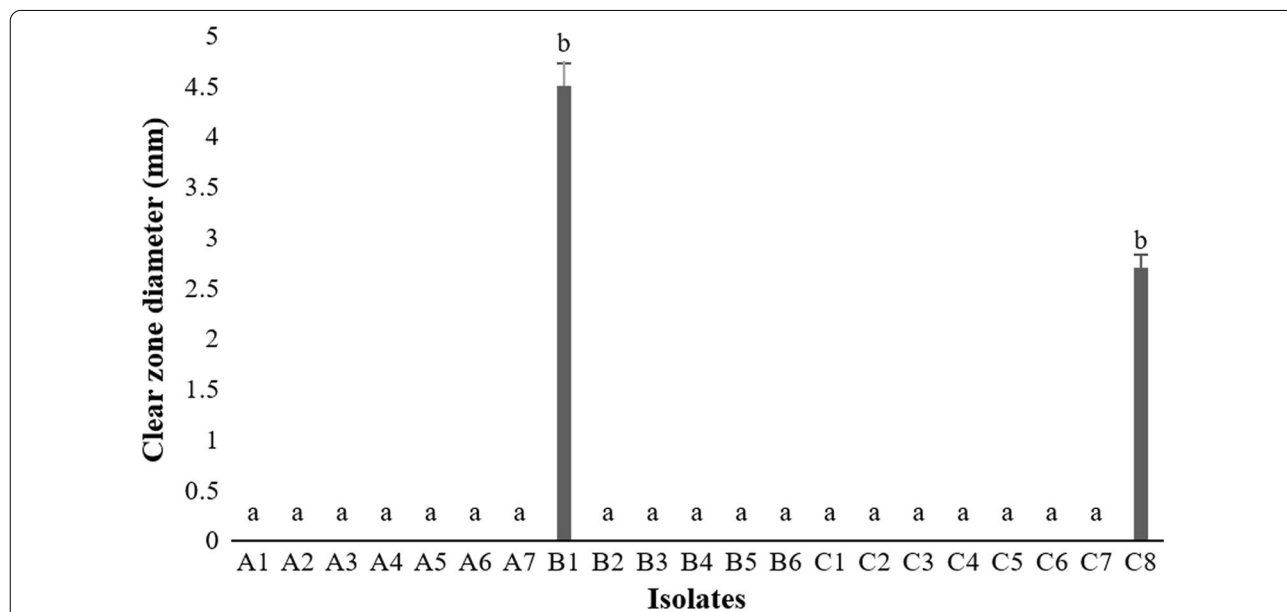
As many as 21 endophytic bacterial isolates were collected from the healthy leaves of Rangpur lime with a total plate count number of  $1.06 \times 10^7$  CFU/g. Most isolates found were rod-shaped Gram-negative bacteria (11 isolates), followed by spherical shape Gram-negative bacteria (6 isolates) and spherical shape Gram-positive bacteria (4 isolates). In this initial screening, only two isolates, namely B1 and C8, demonstrated antagonistic activities against XCC (Fig. 1). These isolates produced a clear zone with the diameter of 4.53 mm and 2.55 mm and were chosen for further assays in this study.

**Antagonistic activity of the selected endophytic bacteria at different incubation times**

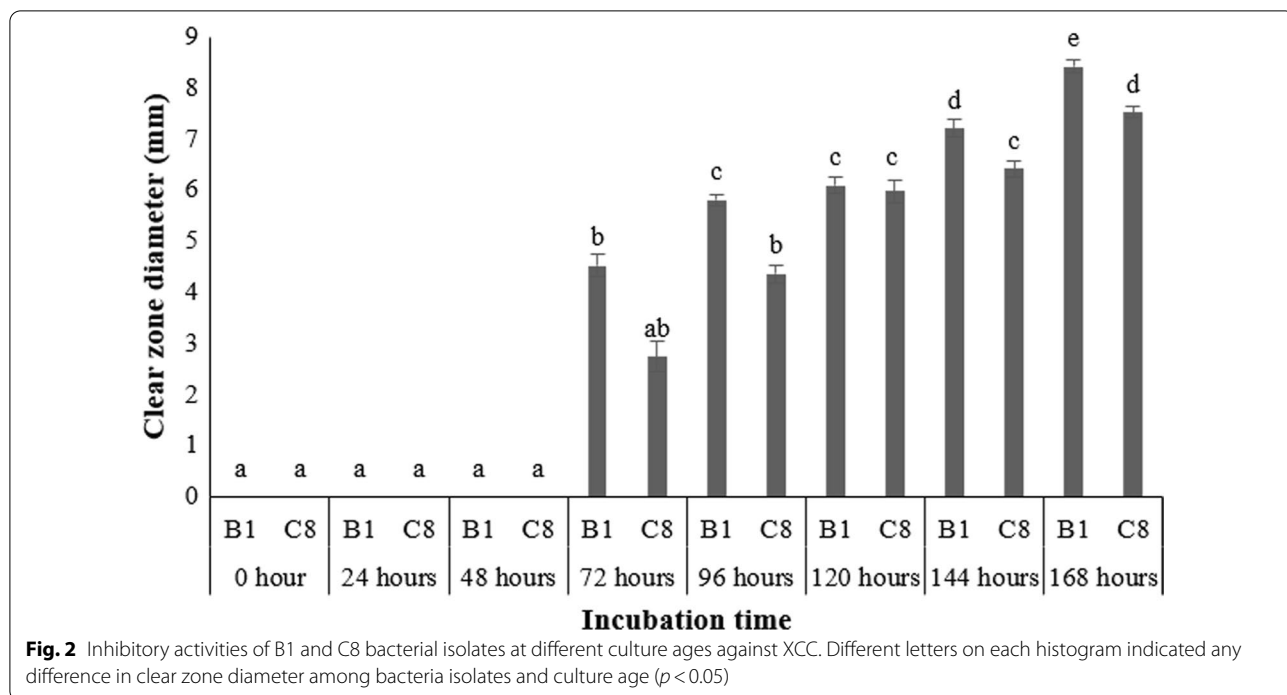
The antagonistic activity of both B1 and C8 demonstrated a linear correlation to the increase in the incubation period. Similar to the initial screening, this activity appeared once it had been incubated for 72 h and its potency increased gradually as the incubation continued ( $p < 0.05$ ) (Fig. 2). The strongest inhibition activity of both isolates was recorded at 168 h of culture ages, with clear zone diameters for B1 and C8 being 8.41 and 7.51 mm, respectively.

**Antagonistic activity of the secondary metabolites**

The secondary metabolites (in the form of CFS) produced by B1 and C8 at 72, 96, 120, 144, and 168 h of culture age were assayed for their inhibitory activity against



**Fig. 1** Inhibitory activities of Rangpur lime endophytic bacteria against XCC. Different letters on each histogram indicated any difference in clear zone diameter among bacterial isolates ( $p < 0.05$ )

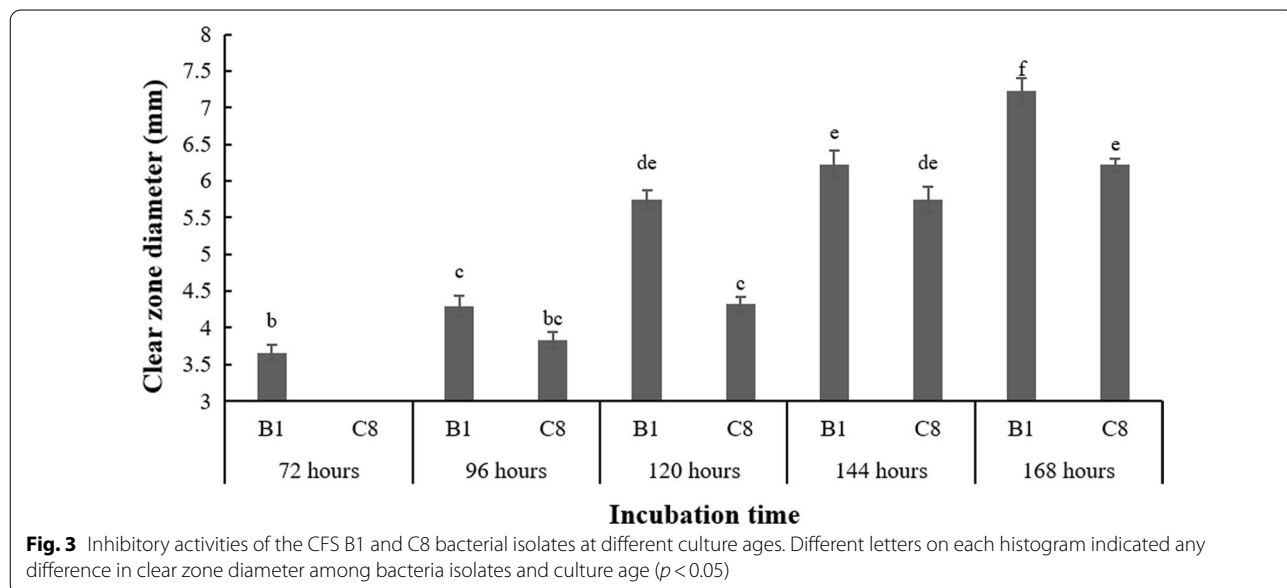


XCC. In this assay, the CFS harvested from both isolates displayed a similar dynamic to its cell suspension counterparts, where the inhibition activity increases progressively according to the increase in culture age ( $p < 0.05$ ) (Fig. 3). The highest inhibition activity from the CFS of both isolates was observed during 168 h of culture age, with a clear zone diameter of 7.23 mm and 6.22 mm, respectively. Overall, the CFS from B1 had a significantly

higher inhibition activity compared to C8. Its activity was already observed when the culture age was only 72 h.

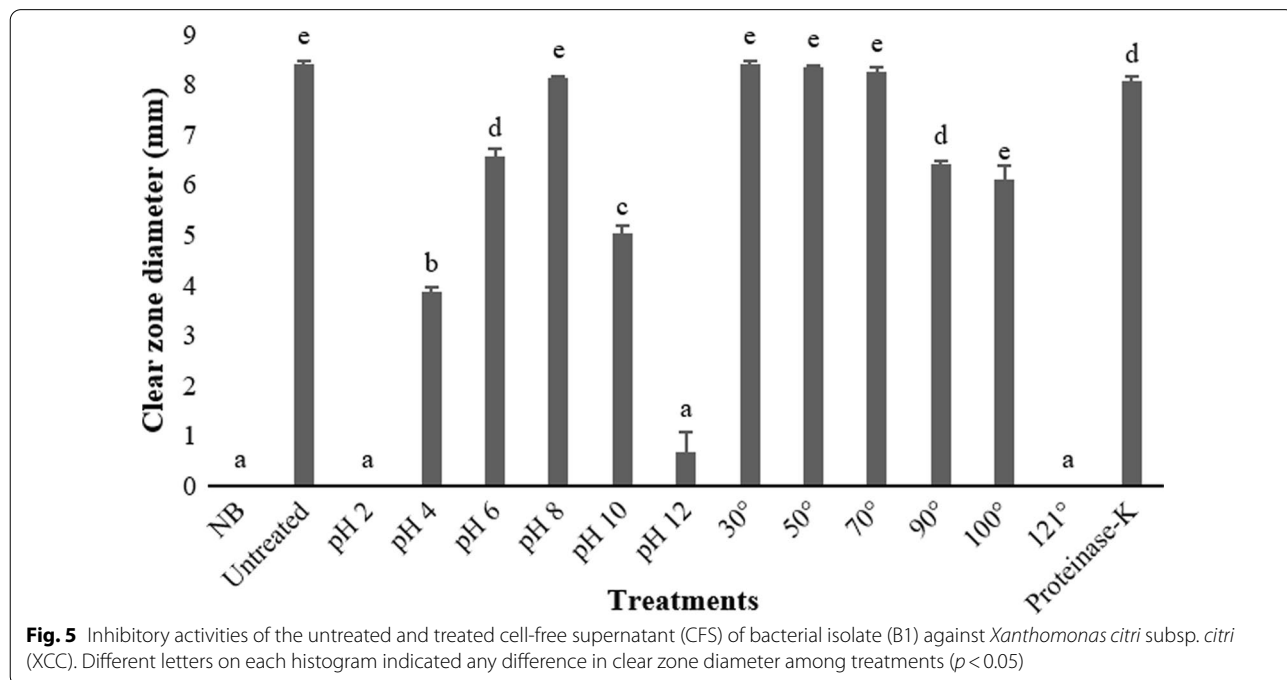
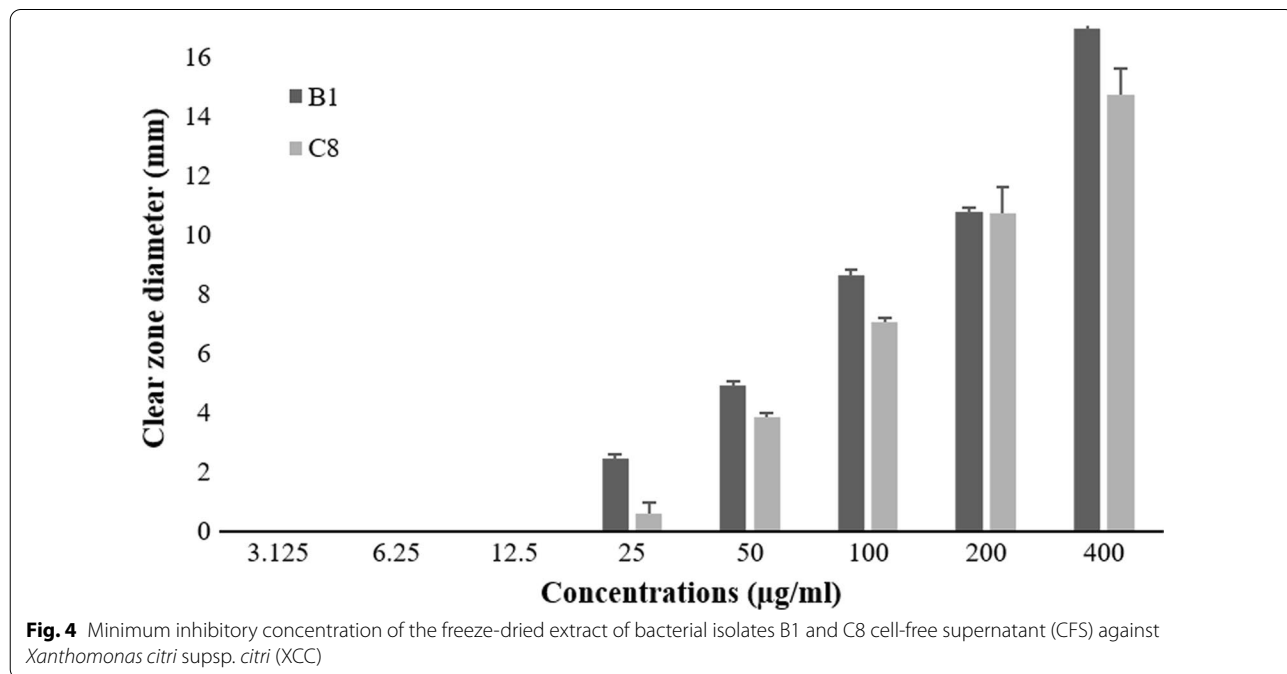
**Minimum inhibitory concentration (MIC) of B1 and C8 secondary metabolites**

MIC is characterized as the minimum concentration required for an antimicrobial agent to inhibit the growth of a target pathogen. Since the strongest



anti-XCC activities of both CFSs were recorded during the final day of incubation, their MIC was evaluated using the agar well diffusion method. Based on the assay, the MIC value of the CFSs harvested from B1 and C8 was 25 µg/ml (Fig. 4).

**Physicochemical characterizations of selected isolates CFS**  
 CFS produced by B1 demonstrated strong stability in the presence of heat. It could withstand a temperature up to 70 °C without significantly losing its inhibitory activity ( $p < 0.05$ ). However, when heated to a high temperature, it gradually loses its inhibitory activity (Fig. 5). It only retained 70–77% of its inhibitory activity when heated



at 90–100 °C and completely lost its inhibitory activity at 121 °C. In terms of pH and proteinase-K stability, the inhibitory activity of B1 CFS appeared to be stable at pH 8 and the presence of proteinase-K (Fig. 6).

Similarly, the CFS of C8 also demonstrated strong stability in the presence of heat and proteinase-K. It could tolerate proteinase-K and 50–70 °C temperatures without significant loss in its inhibitory capacity. Compared to B1, it appeared to display relatively high stability at 90 °C by retaining almost 95% of its previous inhibitory activity ( $p < 0.05$ ). However, it completely lost its inhibitory capacity when heated to a high temperature (Fig. 6). For pH stability, the CFS of C8 was stable at pH 8 and sensitive to pH changes. Its inhibitory capacity experienced significant reductions in high and low pH.

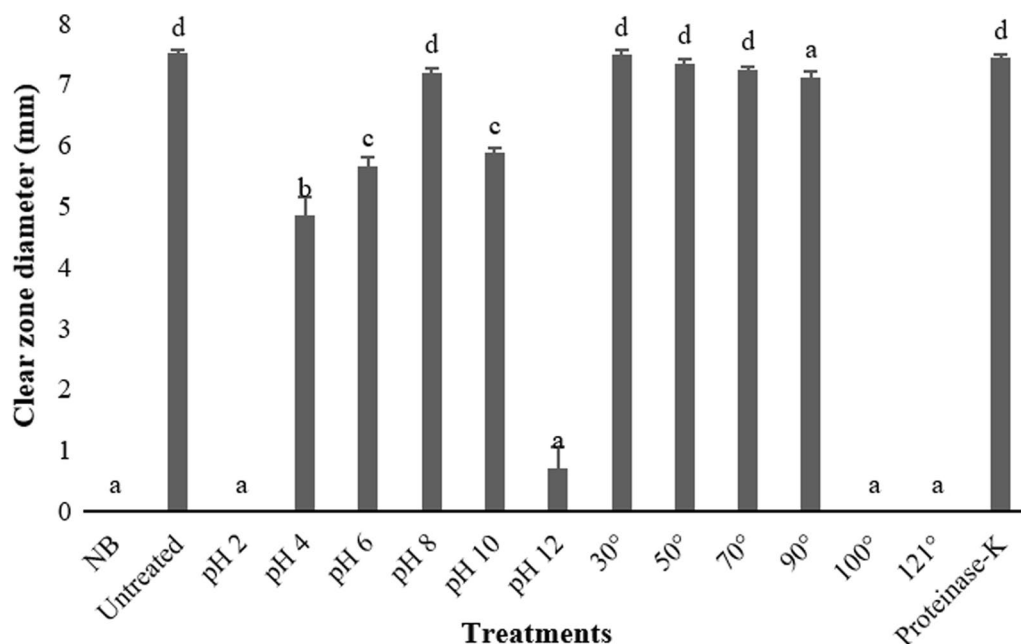
#### In planta antagonistic activity of the cell suspensions and cell-free supernatants of the selected isolates against XCC

All treatments could effectively protect the host plant from XCC infection. Those treatments significantly decrease the disease incidences in the host plant by reducing the number of infected leaves up to 50% by the end of the experiments. CS and CFS treatments of B1 provided better protections from the citrus canker incidence compared to C8. In the end, the percentage of symptomatic leaves in the plants treated with CS and CFS of B1 isolate was only 28%. This was relatively lower

than the plants treated with CS and CFS of C8 that had 37% and 42% of symptomatic leaves, respectively (Fig. 7).

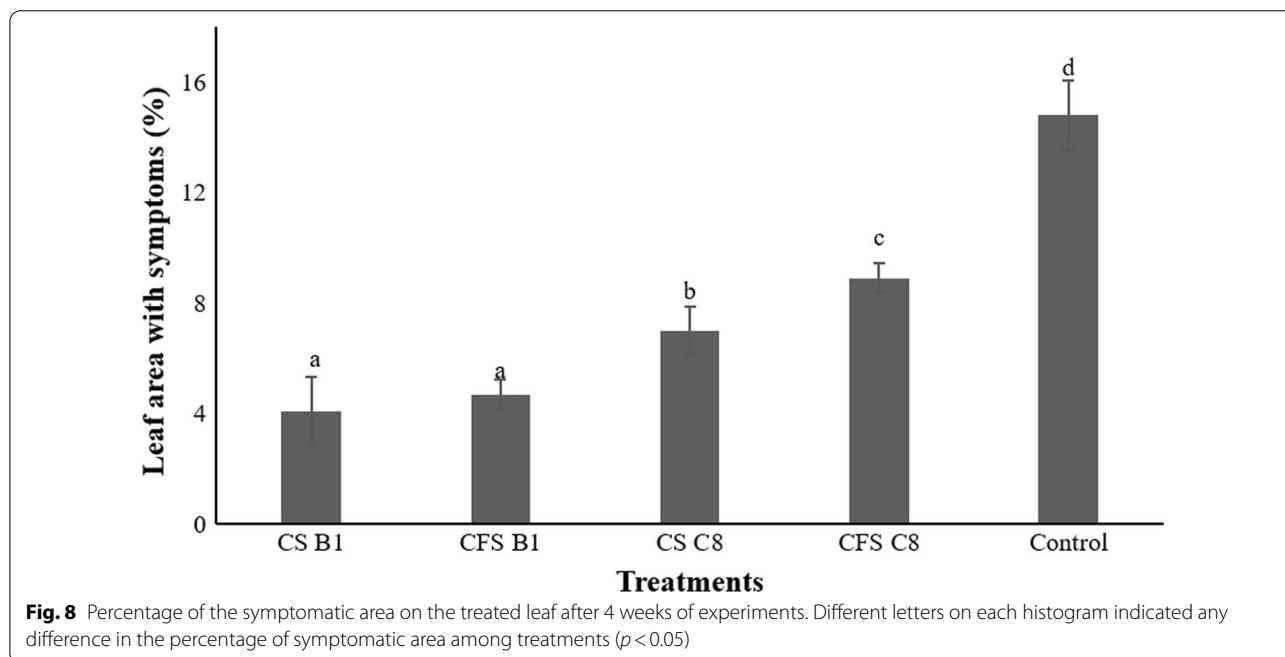
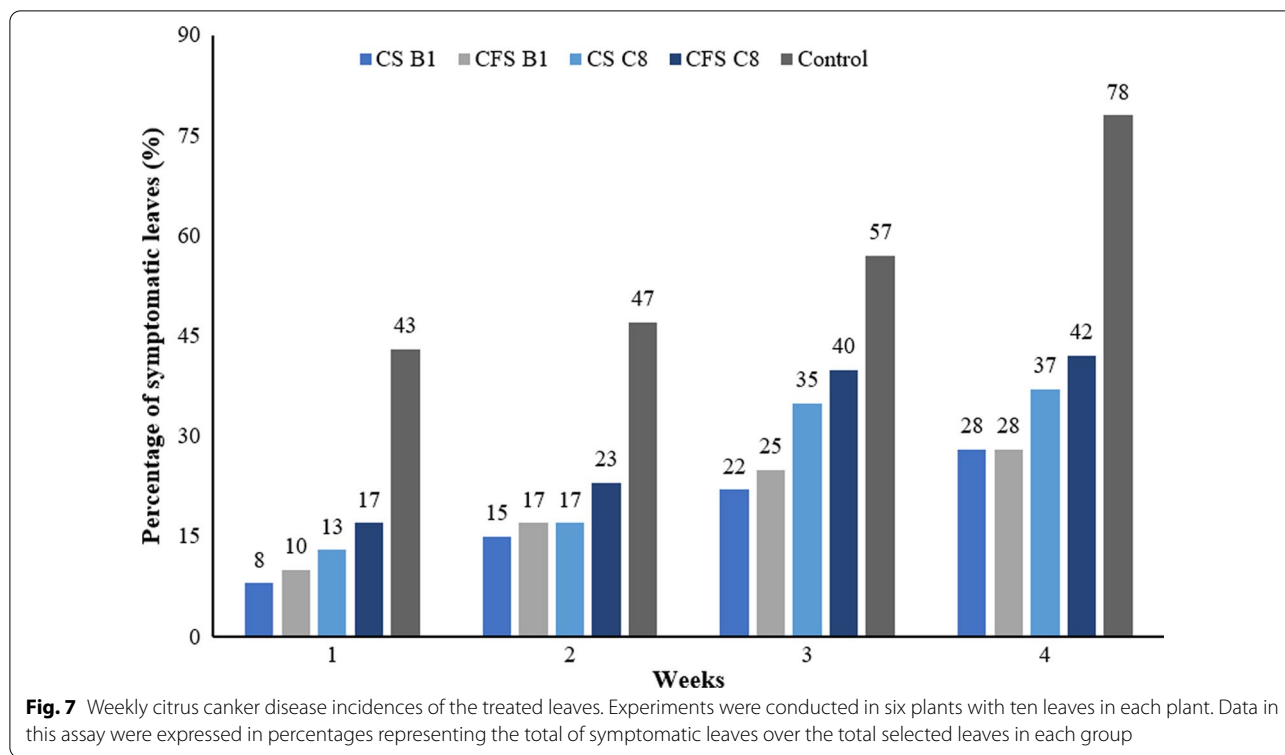
Cell suspension and CFS treatments of both endophytic bacteria could also effectively reduce disease severity in the host plant. Treated plants developed small symptoms areas and few necrotic spots. Consistent with its disease incidences, plants treated with cell suspension and CFS of B1 also had a lower percentage of symptoms area and necrotic spots compared to the plants treated with C8 ( $p < 0.05$ ) (Figs. 8 and 9). Plants treated with cell suspensions and CFS of B1 isolate had 4.11% and 4.7% of symptoms area and 3.4 and 3.8 necrotic spots, respectively. Meanwhile, plants treated with C8 developed bigger symptom areas and higher necrotic spots with 7–9% and 4–7% necrotic spots, respectively.

The early signs of citrus canker are the appearance of small and brown necrotic spots or lesions on the lower side of the leaves caused by cell hyperplasia and tissue necrosis. As the infection progresses, the lesion grows in size, becomes darkened, and thickens, a chlorotic halo around the lesion appears, and both epidermal sides of the infected leaf become ruptured as a result of continuous tissue necrosis (Gottwald et al. 2002). In this study, treated leaves had visually small and less lesions. Lesions on the treated leaves showed a small increase in diameter and tended to develop small chlorotic halo around the lesions. In contrast, the control leaves tend to develop big and high numbers of lesions. At the end



**Fig. 6** Inhibitory activities of the untreated and treated cell-free supernatant (CFS) of bacterial isolate C8 against XCC. Different letters on each histogram indicated any difference in clear zone diameter among bacteria isolates and culture age ( $p < 0.05$ )

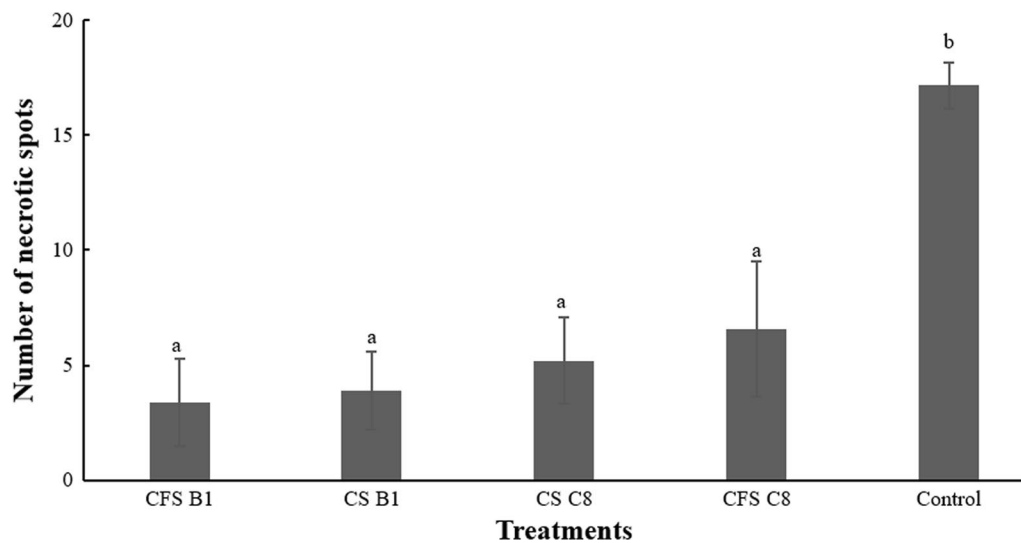




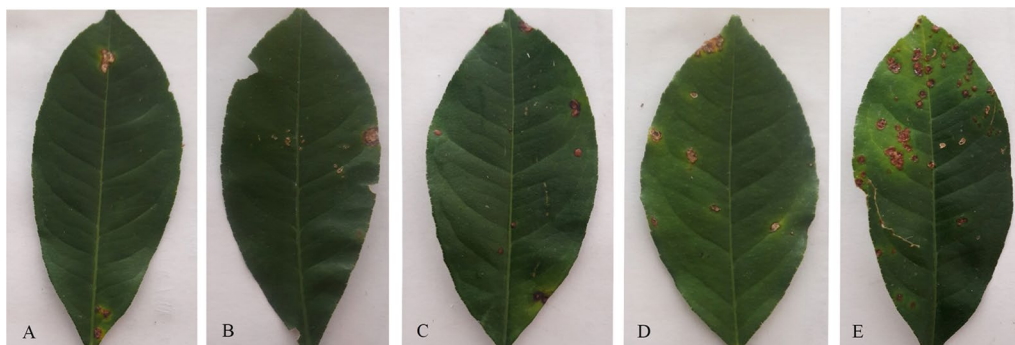
of the experiment, the lesions on the control leaves were big, brownish, and corky with a big chlorotic halo around them (Fig. 10).

**Identification of selected endophytic bacteria**

The phylogenetic tree showed that B1 isolates were identified as *Staphylococcus pasteurii* with 100% similarity (Fig. 11). C8 was identified as *Staphylococcus*



**Fig. 9** Number of necrotic spots on the treated leaf after 4 weeks of experiments. Different letters on each histogram indicated any difference in the number of necrotic spots among treatments



**Fig. 10** Citrus canker symptom development on the final day of the experiment on Rangpur lime leaves treated with *Xanthomonas citri* subsp. *citri* (XCC) and biocontrol agents. **A** Leaf treated with the cell suspension (CS) of B1 and XCC, **B** leaf treated with the cell-free supernatant (CFS) of bacterial isolate (B1), **C** leaf treated with the CS of bacterial isolate (C8), **D** leaf treated with the CFS of C8, **E** leaf treated with XCC only (control)

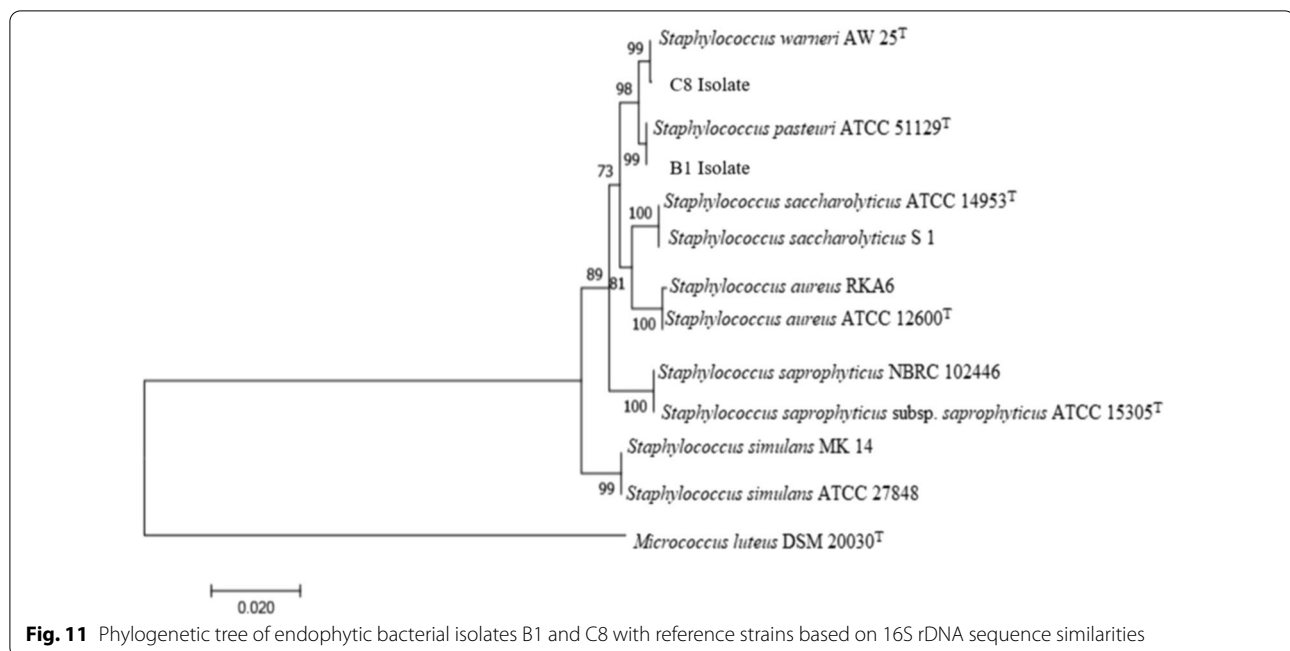
*warneri* with 99.98% similarity. According to Drancourt et al. (2000), bacterial isolates can be classified into the same species if it shares  $\geq 97\%$  similarity, and into the same strain if it shares  $\geq 99\%$  similarity on 16S rDNA sequences.

## Discussions

In the present study, 21 bacterial isolates from the leaves of RL plants were collected. Among all bacterial isolates, two (B1 and C8) isolates displayed a strong antagonistic activity against the target phytopathogen. Unlike most biocontrol bacteria, the inhibitory activities of these isolates only started to appear once they had been incubated for at least 72 h. Their activity also experienced a

significant gradual increase as a response to a prolonged incubation period. However, compared to other biocontrol bacteria, their activity appeared relatively late. Previously, most of the antagonistic activity against *Xanthomonas* species usually appeared during the 24–48 h of culture age. Al-Saleh (2014) reported that the inhibitory activity of *Pseudomonas fluorescens* from Saudi Arabia against XCC has already appeared during the 24 h culture age.

According to the in vitro antagonistic assay of B1 and C8, the strongest inhibitory activities were recorded at 168 h of culture age with an inhibition zone diameter of 8.42 mm and 7.52 mm, respectively. Similar findings were also found in another related study where



**Fig. 11** Phylogenetic tree of endophytic bacterial isolates B1 and C8 with reference strains based on 16S rDNA sequence similarities

the highest inhibition activity of endophytic *Bacillus subtilis* LE024 and *B. amyloliquefaciens* LE109 isolated from *C. maxima*, *C. aurantifolia* was 7.8 and 8.2 mm, respectively (Daungfu et al. 2019). This finding may indicate that the primary mode of action of B1 and C8 in controlling XCC is the production of secondary metabolites, that are related to their growth dynamics. According to Ruiz et al. (2010), the growth dynamic among bacterial species is entirely exclusive and its differences are heavily influenced by the nutrient uptake and proliferation rate. As a result, some bacterial species may take a long time to enter their log or stationary phases, therefore affecting the overall production rate of secondary metabolites.

Secondary metabolites production is one of the most substantial antibiosis mechanisms used by endophytic bacteria to control certain pathogens in their host (Arguelles-Arias et al. 2009). In the present study, the secondary metabolites (in the form of CFS) of B1 and C8 isolates demonstrated promising antagonistic activity against XCC. Consistent with the cell suspensions, their CFS also experienced significant gradual increases in inhibitory activities as a result of the extended incubation period. The highest inhibitory of the CFS from both isolates was also recorded at 168 h of culture age with 7.23 and 6.22 mm of clear zone diameter, respectively. This finding indicated that during the incubation periods before harvest, both B1 and C8 isolates were still actively producing their secondary metabolites. Thus, by the end of the experiment, there might be an

accumulation of secondary metabolites within the culture, which results in strong inhibitory activity.

Previous studies on the inhibitory activity of the crude CFS of promising bacteria against XCC are very limited. However, studies on other *Xanthomonas* species are relatively abundant. Licheva et al. (2013) found that seven *Bacillus* strains isolated in Bulgaria could secrete CFS substances that could inhibit the growth of *Xanthomonas vesicatoria* with clear zone diameters ranging between 4 and 22 mm. Chaisit et al. (2010) reported that *Bacillus amyloliquefaciens* KPS46 also demonstrated an antagonistic activity against *X. axonopodis* in a lawn culture with a clear zone diameter up to 7.1 mm.

In a further investigation, it was found that MIC values of the CFSs produced by B1 and C8 were 25 µg/ml. These values are relatively lower than the values found in several related studies. Previously, Rabbee et al. (2019) also found that the MIC value of endophytic *Bacillus velezensis* ethyl acetate CFS extract against XCC was 46.9 µg/ml. In addition, it was also revealed that the inhibitory activities of both CFS were not affected by the presence of proteinase-K and a wide range of temperatures. However, their inhibitory activities were not stable in highly acidic or alkaline conditions. Both CFSs tended to gradually lose their inhibitory activity as they were adjusted to pH conditions far from their initial pH. Nevertheless, these findings may still indicate that both CFS may still be suitable for industrial purposes. Heat stability properties possessed by both CFSs are beneficial for industrial application and handling.

In the *in vivo* antagonistic tests, the cell suspensions and CFS of both isolates were applied in the leaves of healthy Rangpur lime plants as the preventive treatment, before being challenged with XCC. It was found that the preventive treatment of cell suspensions and CFS have given effective protections against XCC by significantly reducing the disease severity and incidence, shown by a significantly lower weekly disease incidence, necrotic spots, and percentage of leaf area with symptoms. Visually, leaves treated with cell suspensions and CFS also tend to be healthier as they develop fewer symptoms compared to the control leaves.

Overall, cell suspensions of both isolates displayed better host protections against XCC compared to its CFS counterpart. Leaves treated with cell suspensions had low disease incidence and developed less severe symptoms. A similar outcome was reported by Li et al. (2012) that found that the preventive treatment of *B. amyloliquefaciens* cell suspension was very effective to protect anthurium plants against *X. axonopodis* pv. *dieffenbachiae* (XAD). The effectiveness of cell-based biocontrol systems in preventive treatments could be related to the ability of the biological control agent to rapidly proliferate and colonize the host's tissue (Latz et al. 2018). Once within its host, biocontrol agents such as bacterial endophytes will occupy the available ecological niche and lurk nutrients that can be used by the pathogens. Moreover, this association can also elicit continuous secretions of the endophyte's secondary metabolites within their host that are harmful to the pathogens (Fadiji and Olubukola 2020).

In contrast to cell-based biocontrol systems, the CFS or other chemical compounds such as antibiotics and fungicides when applied to the plants tend to be adsorbed and distributed by the plants via vascular tissue. This distribution causes a decrease in concentrations of the chemicals in the targeted area and affects the overall inhibitory activity (Agrios 2009). Comparable to chemical pesticides such as copper spray and antibiotic spray, CFS's capacity to protect the host can be easily weakened by external disturbances such as watering and wind-driven rain. Exposures to these factors can potentially reduce the CFS concentrations or even sweep it from the targeted area (Gottwald and Timmer 1995). However, the CFS-based biocontrol systems may still be effective if used in curative treatments. Li et al. (2012) found that the curative regiment of CFS obtained from *B. amyloliquefaciens* was very effective to protect anthurium plants against *X. axonopodis* pv. *dieffenbachiae* (XAD). Therefore, it would be necessary to assess the curative potencies of the CFS alongside its CS for future study.

Based on the phylogenetic identification, B1 and C8 were identified as *Staphylococcus pasteurii* and *Staphylococcus warneri*, respectively. This finding may be the

first report of the antagonistic activity of *S. pasteurii* and *S. warneri* against XCC. Previously, only several genera of bacteria could inhibit XCC's growth, namely *Bacillus* and *Pseudomonas* (Michavila et al. 2017). Currently, *S. warneri* and *S. pasteurii* are mostly known as human microflora and are rarely known to cause disease. However, they can still infect humans with a compromised immune system (Toltziz 2018). This possesses a challenge to the marketability and the safety of these agents when used by the farmer in the field. To safely use these agents, we can utilize their CFS which can be produced through a fed-batch fermentation where the bacterial cells are grown to a specific phase (Srivastava et al. 2010). Some of the known antimicrobial substances produced by both bacteria are pastereucin and warnericin (Hong et al. 2018).

## Conclusions

*Staphylococcus pasteurii* and *S. warneri* isolated from Rangpur lime had the potency as biological control agents of XCC. They demonstrated strong *in vitro* and *in planta* antagonistic capacity against XCC. Both isolates could be recommended to use as alternatives to chemical treatments in preventing citrus canker.

## Abbreviations

RL: Rangpur lime; CC: Citrus canker; XCC: *Xanthomonas citri* Subsp. *citri*; CFU: Colony-forming unit; CS: Cell suspension; CFS: Cell-free supernatant; MIC: Minimum inhibitory concentration; NA: Nutrient agar; NB: Nutrient broth; ANOVA: Analysis of variance; OD: Optical density; PCR: Polymerase chain reaction; rpm: Rotations per minute; BLAST: Basil local alignment tools; NCBI: National Center for Biotechnology Information.

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## Author contributions

YAN contributed to investigations, writing, editing, and data analysis; SS and SW helped in supervisions, methodology, and writing—review. All authors have read and approved the final manuscript.

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## Availability of data and materials

The datasets used and analyzed during this project are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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